Histological and chemical changes in tannic compounds of evergreen oak leaf litter

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In evergreen oak leaves collected at a western Mediterranean site (Var, France), the amounts of both hydrolyzable and condensed tannins decrease through the depth of the leaf litter. The decrease in condensed tannins could be partly explained by the cellular degradation of the lower epidermis and the mesophyll of leaves, as observed in histological sections. However, the condensed tannins may also be degraded by microbial enzymes because their average molecular weight decreased by about 50%. In contrast, the decrease in hydrolyzable tannins seems to be initiated by microbial enzymatic action, which is then followed by the same degradation process described above.

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La teneur en tanins hydrolysables et condensés dans les feuilles de chêne vert, prélevées dans une station de Méditerranée occidentale, diminue avec la profondeur des feuilles dans la litière. Pour les tanins condensés, la désintégration cellulaire observée sur les coupes histologiques au niveau de l'épiderme inférieur et du mésophylle semble être la cause essentielle de cette diminution; cependant, la réduction de 50% de la masse moléculaire moyenne de ces composés permet d'envisager également une action enzymatique d'origine vraisemblablement microbienne. Pour les tanins hydrolysables, cette diminution semble pouvoir être attribuée tout d'abord à une action enzymatique microbienne et ensuite à l'action mécanique déjà évoquée pour les tanins condensés.

Introduction

Evergreen oak (*Quercus ilex* L.) forms characteristic forest climax populations widely distributed in the western Mediterranean area (Lossaint and Rapp 1978). Its litter is composed of up to 48% leaves and 52% branches, twigs, flowers, and fruits (Rapp 1969). Several studies have been undertaken to examine the role of microorganisms (Chabert *et al.* 1984) and microarthropods (Poinsot-Balaguer and Tabone 1985; Poinsot-Balaguer and Tabone 1986) in the litter decay. Other studies have dealt with the microflora that grows on the leaf surface of various plants (Preece and Dickinson 1971; Dickinson and Preece 1976). In litter, Jensen (1974) showed that microorganisms could degrade up to 80% of the plant material. The degradation of pine needles (Gourbière 1981), hornbeam leaves (Mouchacca and Geoffroy 1984), and sycamore (*Acer pseudoplatanus*) leaves (Rodger and Blakeman 1984) is initiated by microflora within the canopy, which contains a great number of microorganisms, especially during its senescence.

Nevertheless, when a plant structure contains secretion elements (Metge and Girardin 1980; Crawford 1981) that are important defensive chemicals, such as tannins (Perry 1985), inhibition effects may appear against insects and microorganisms (Kühnelt 1963; Feeny and Bostock 1968). The repartition of these polyphenolic compounds in the plant as well as their concentration are two important factors for the limitation or the selection of organisms involved in leaf biodegradation. Indeed, as reported by several authors (Harrison 1971; Preece and Dickinson 1971; Campbell 1985), the presence of tannins affects litter decay rate. Thus, Benoit *et al.* (1968) showed tannin effects on degradation of plant organic

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compounds, while Hatway and Seakins (1958) and Benoit and Starkey (1968) demonstrated these effects on carbohydrate decomposition. On the other hand, Satchell and Love (1967) have shown that tannin degradation by microorganisms is associated with an increase in the earthworm population in the litter.

Although the localization and the composition of tannins in leaves have been examined in many plant species, especially in *Quercus* spp. (Feeny and Bostock 1968; Santappa and Rao 1982), they were never studied in evergreen oak (*Quercus ilex* L.).

In the present work, evergreen oak leaf degradation after leaf drop was followed by histological and chemical studies, with a focus on the disappearance of tannins. Four different stages of leaves were investigated: 1-year-old leaf; 2-year-old leaf; whole leaf typical of the litter upper layer (L_1); and leaf still recognizable in spite of decay, constituting the litter underlayer (L_2).

This study represents part of a larger research project concerning the degradation of evergreen oak litter by microorganisms and microarthropods.

Materials and methods

Sampling station

Leaves were collected in July 1985 from various trees growing in dense copses planted in La Gardiole de Rians (Var, France). The copses are about 35 years old and form a continuous cover. The station climate was described by Loisel (1976).

Histological study of the leaves

Leaf thin sections (5 to 15 μm) were obtained by using a microtome (Leitz 512). Microscopic observations were performed on thin-section series. After removal of paraffin, thin sections were fixed in a 5% ferrous alum solution and successively stained by hematoxylin for nucleus and cytoplasm, by safranin for suberified and lignified walls, and by aniline blue for cellulosic walls. Tannin compounds stained orange–red.

After treatment, the sections were cleared by a salicylate solution, washed with xylene, and mounted in Canada balsam on microscope slides.

To confirm the location of the isolated tannin cells, thin sections were also prepared from living material. In secretory cells, tannins appeared dark blue with ferric chloride and brown with potassium bichromate (Locquin and Langeron 1978).

Extraction and chemical analyses of tannins

Leaves were washed, dried, and then treated according to the method of Broadhurst and Jones (1978) to separate tannins from other plant compounds. Hydrolyzable tannins were separated from condensed tannins by ethyl acetate extraction. Hydrolyzable and condensed tannins were then separately purified according to the method of Hagerman and Butler (1980) by liquid chromatography on a column (30 × 2 cm) of Sephadex LH 20. The eluant flow was maintained at 0.8 mL min^{-1} by means of an Analtrap EC 93-TM pump. The eluted compounds were detected with a Pye Unicam ultraviolet-visible detector. An initial elution with ethanol removed proteins and other contaminants. Tannins were then eluted with dioxane–water (45:55, v/v) with detection at 540 nm. At this wavelength the specific extinction coefficients of tannins are low ($E = 0.12$ for condensed tannins, $E = 0.072$ for hydrolyzable tannins), but no contaminating compounds interfere.

In the various fractions, the absence of proteic and inorganic compounds, as well as the oxidation state of tannin molecules, was checked by microanalyses of C, H, N, and ash. The average molecular weight (MW) was measured by vapour pressure with an osmometer. These methods made it possible to establish the average empirical formula (EF) and the unsaturation number (UN) for each tannin type. Infrared spectra of the purified products were recorded with a 298 Perkin–Elmer spectrophotometer using KBr pellets.

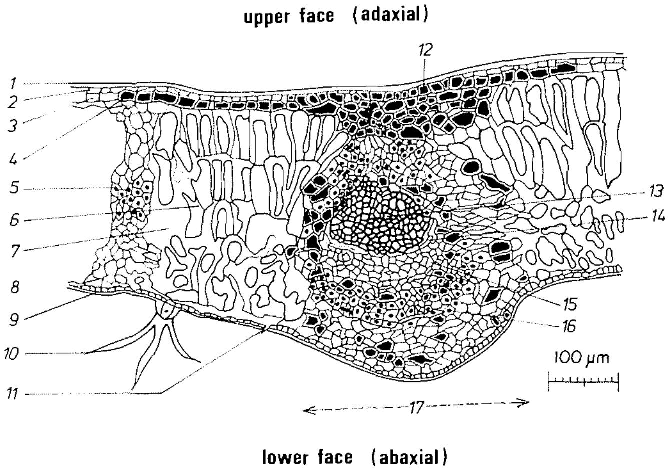

FIG. 1. Thin section of a 1-year-old leaf. 1, upper cuticle; 2, upper epidermis; 3, hypodermis; 4, tannin cells; 5, sclereid; 6, parenchymatic palisade; 7, lacunar parenchyma; 8, lower epidermis; 9, lower cuticle; 10, tufted hair; 11, stoma; 12, sclerenchyma; 13, secondary xylem; 14, ligneous phloem cambium; 15, secondary phloem; 16, sclerous fiber canopy; 17, central vein.

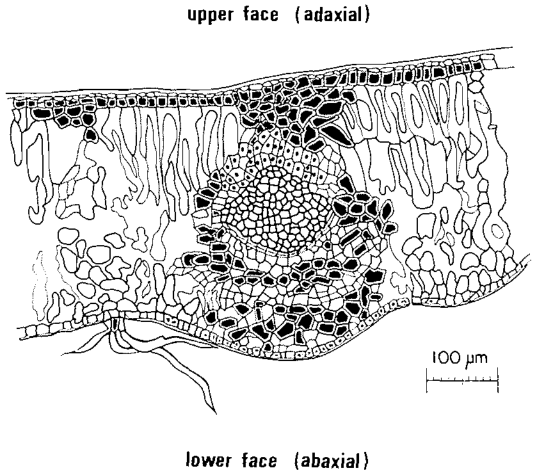

FIG. 2. Thin section of a 2-year-old leaf. (For explanation of areas see legend in Fig. 1 caption.)

The detection of the different monomers within specific fractions was performed by silica-gel thin-layer chromatography (TLC), using ellagic acid (Aldrich), gallic acid, and catechin (Fluka) as reference substrates and chloroform – ethyl acetate – formic acid (50:40:10, by volume) as solvent. The compounds were detected under ultraviolet light (254 nm) and after spraying with a $FeCl_3$ (1%) aqueous solution; tannins, gallic acid, and ellagic acid are characterized by a deep-blue color and catechin by a green–blue color.

Results

Changes in evergreen oak leaf structure

Sections of both 1- and 2-year-old leaves (Figs. 1 and 2) were consistent with typical leaf anatomy. Tannin cells were observed within the sclerenchyma and the lower epidermis in both ages of leaves but were mainly concentrated in the upper epidermis. However, in the 2-year-old leaf, tannin cells were more numerous and larger than those in the 1-year-old leaf.

The surface of the L_1 leaf (Fig. 3) was largely unchanged

TABLE 1. Characteristics of condensed tannin in the different leaf stages examined

	1 year old	2 years old	L_1	L_2
%	1.873	2.144	2.647	0.39
MW	2622	3258	4179	2296
EF	$C_{4.135}H_{3.910}O_{2.904}$	$C_{4.149}H_{4.405}O_{2.863}$	$C_{4.211}H_{4.156}O_{2.832}$	$C_{4.152}H_{3.875}O_{2.894}$
UN	3.18	2.947	3.133	3.215

NOTE: %, percent tannin concentration of leaf dry weight; MW, average molecular weight measured by osmometric method; EF, empirical formula estimated from microanalysis results; UN, unsaturation number = (2C + 2 − H)/2.

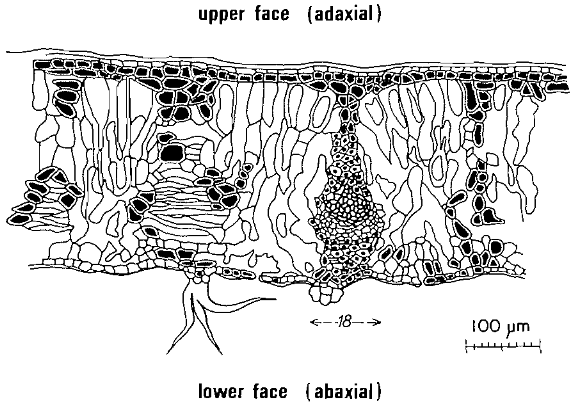

FIG. 3. Thin section of a leaf from the L_1 litter stage. Cellular contents are less dense than in both 1- and 2-year-old leaves. 18, secondary vein. (For other areas see legend in Fig. 1 caption.)

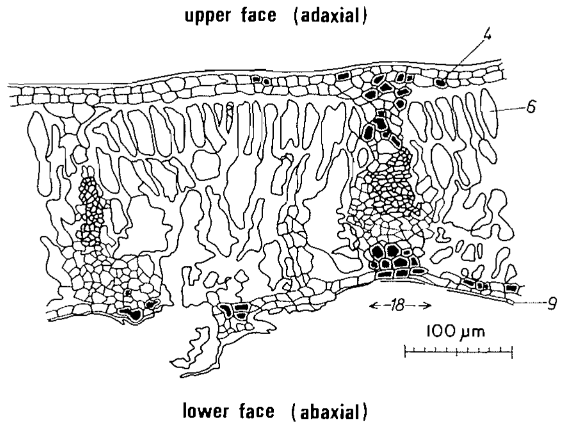

FIG. 4. Thin section of a leaf from the L_2 litter stage. Parenchyma cells (6) are empty, the tannin cells (4) are not abundant, and there are no tufted hairs. The lower cuticle (9) is cut into strips. (For explanation of areas see legend in Fig. 1 caption.)

when compared with that of the 2-year-old leaf. The lower and upper cuticles, as well as the tufted hairs, were undamaged. However, the contents of the parenchymatous cells had a lower density and the tannin cell number was lower.

By contrast, in the L_2 leaf (Fig. 4), the entire mesophyll as well as the lower and the upper epidermis had lost their cell contents. The lower cuticle was cut into strips in places and bristles had disappeared. Only a few tannin cells remained.

These observations suggest that evergreen oak leaf degradation occurs initially through the lower epidermis and that the lamina are disrupted before the upper cuticle.

Chemical analysis of tannins

After purification, condensed and hydrolyzable tannins were obtained as a light-sensitive, fine brown powder (Tables 1 and 2).

Table 1 shows an increase in the percentage of condensed tannins in the leaf (dry weight), from the 1-year-old leaf to the L_1 stage. In contrast, the value obtained in L_2 represents only 14% of the maximum value measured in L_1 leaves. Moreover, the MW value increases from the 1-year-old leaves to the L_1 stage, suggesting a progressive molecular condensation. It then decreases about 50% from the L_1 to the L_2 stage. This result implies that condensed tannin degradation could be initiated by an hydrolysis between two monomers. However, this cannot explain the low overall level of tannin compounds in the L_2 leaf; it must instead be attributed to cellular decomposition as observed in the histological study.

The concentration of hydrolyzable tannins (Table 2) is maximal in the 2-year-old leaf. It decreases in all the litter leaves, declining to about 12% of the highest concentration in the L_2 leaf. The MW values as well as the UN values of these tannins do not vary significantly through the different stages of leaf decomposition. However, the decrease in the L_1 leaf content of hydrolyzable tannins (39.9%) cannot be explained by cellular decomposition, since this is not important until the L_2 stage, as shown for condensed tannins and by the histological study. The decrease could arise from either a total degradation or a partial oxidation of the molecules, which would not be detected by the analytical procedures used.

The aromatic monomers gallic acid, ellagic acid, and catechin could be detected in the four leaf stages by thin-layer chromatography, but the presence of these compounds could result from the leaf metabolism rather than from tannin degradation.

Discussion

Histological study indicates that changes in evergreen oak leaves during senescence and degradation in the upper litter zone seem to be characterized by a slow disorganization of the mesophyll and lower epidermis. Observations of some mycelia inserted in the degenerating cells (F. Leonardon, personal communication) suggest that cell disruption could be caused by cellulolytic or ligninolytic exoenzymes produced by microorganisms. Microbial enzymes could also degrade hydrolyzable tannins, but the disappearance of bulk tannins, particularly condensed tannins, probably occurs by physical loss during the cell disintegration.

Histological study of thin sections of the 1-year-old leaf (Fig. 1) indicated that the leaf degradation may begin on the underside of the leaf because the upper cuticle is thicker and the hypodermis contains numerous tannic cells. The upper surface thus constitutes a more substantial barrier to microorganisms. The disintegration of lower epidermal cells and mesophyll cells would allow a microbial overgrowth of the leaf and a mechanical release of tannins. The enzymatic potential

TABLE 2. Characteristics of hydrolyzable tannin in the different leaf stages examined

	1 year old	2 years old	L_1	L_2
%	0.382	0.537	0.323	0.067
MW	2330	2263	2331	ND
EF	$C_{4.243}H_{4.095}O_{2.812}$	$C_{4.370}H_{4.280}O_{2.705}$	$C_{4.328}H_{4.568}O_{2.718}$	ND
UN	3.196	3.230	3.044	ND

NOTE: %, percent tannin concentration of leaf dry weight; MW, average molecular weight measured by osmometric method; EF, empirical formula estimated from microanalysis results; UN, unsaturation number = (2C + 2 − H)/2. ND, not determined.

of these microorganisms in degradation of tannic compounds has been analyzed in a parallel study.

The data obtained by Jacob and Pignal (1975), Grant (1976), Ganga *et al.* (1977), Mallika and Dhar (1980), Pourrat *et al.* (1985), Deschamps and Lebeault (1984), and Deschamps (1985) suggest that the decrease in hydrolyzable tannin concentrations after the L_1 stage and the decrease of MW for condensed tannins in the L_2 stage are the result of enzymatic action. Furthermore, the disintegration of evergreen oak leaf could be enhanced by mechanical action of fauna unless the latter are too sensitive to tannins. In fact, tannins constitute poisonous substances for collembola beyond a threshold concentration (Sadaka 1986). It would be interesting to know the exact relationship between the degradation potential of these organisms and the tannic compound concentration in evergreen oak leaves.

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